Targeted Gene Expression Using the *GAL4/UAS* System in the Silkworm *Bombyx mori*

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ABSTRACT

The silkworm *Bombyx mori* is one of the most well-studied insects in terms of both genetics and physiology and is recognized as the model lepidopteran insect. To develop an efficient system for analyzing gene function in the silkworm, we investigated the feasibility of using the *GAL4/UAS* system in conjunction with *piggyBac* vector-mediated germ-line transformation for targeted gene expression. To drive the *GAL4* gene, we used two endogenous promoters that originated from the *B. mori* actin A3 (*BmA3*) and fibroin light-chain (*FiL*) genes and the artificial promoter *3xP3*. GFP was used as the reporter. In initial tests of the function of the *GAL4/UAS* system, we generated transgenic animals that carried the *UAS-GFP* construct plus either *BmA3-GAL4* or *3xP3-GAL4*. GFP fluorescence was observed in the tissues of GFP-positive animals, in which both promoters drove *GAL4* gene expression. Animals that possessed only the *GAL4* gene or *UAS-GFP* construct did not show GFP fluorescence. In addition, as a further test of the ability of the *GAL4/UAS* system to drive tissue-specific expression we constructed *FiL-GAL4* lines with *3xP3-CFP* as the transformation marker. *FiL-GAL4* × *UAS-GFP* crosses showed GFP expression in the posterior silk gland, in which the endogenous *FiL* gene is normally expressed. These results show that the *GAL4/UAS* system is applicable to *B. mori* and emphasize the potential of this system for controlled analyses of *B. mori* gene function.

TRANSGENIC organisms are powerful tools for the analysis of gene function. The application of transgenic methods to insects was limited to *Drosophila melanogaster* until recently, mainly because the transposon vector *P* element, which is used for the transformation of *D. melanogaster*, has very strong species specificity. Thus, germ-line transformation using the *P* element has been restricted to species that are closely related to *D. melanogaster* (Handler *et al.* 1993). Recently, several different types of transposons, such as *piggyBac*, *Hermes*, *Minos*, *hobo*, and *mariner*, have been identified in insects and have been used successfully as vectors for germ-line transformation in various insect species (Handler 2001).

The domesticated silkworm (*Bombyx mori*) is one of a few lepidopteran species that have been used for genetic analysis. Hundreds of different geographical and mutant strains have been preserved in Japan, China, Korea, India, Italy, France, and other countries. Among these strains, >200 mutant genes have been identified. These mutants have been used to construct a linkage map (Doira 1992) and to analyze gene function (Nagata *et al.* 1996; Komoto 2002; Matsunaga and Fujiwara 2002; Quan *et al.* 2002). Moreover, a silkworm genome research program is currently underway. Three bacterial artificial chromosome libraries have been constructed from the silkworm genome (*e.g.*, Wu *et al.* 1999), and a silkworm whole-genome sequencing project is about to start. Molecular linkage maps have also been constructed (Promboon *et al.* 1995; Yasukochi 1998; Hara *et al.* 2001; Tan *et al.* 2001; Kadono-Okuda *et al.* 2002), and these maps will be upgraded as further information becomes available from genomic analyses. The expressed sequence tag (EST) database, which includes >60% of the silkworm genes (K. Mita, personal communication), is currently available (SilkBase: http://www.ab.a.u-tokyo.ac.jp/silkbase/), and cDNA microarrays have been produced from 6000 ESTs (K. Mita, personal communication). Moreover, as an experimental animal the silkworm has the advantages that it is easily handled, the larvae are highly adapted for artificial rearing, and the adult moths are unable to fly. Thus, the silkworm is regarded as a model insect for the Lepidoptera in particular. However, since transformation of silkworms was

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not achieved until recently, its utility for gene functional analyses was limited.

In 2000, we developed a germ-line transformation method for the silkworm using the transposable element *piggyBac* as the vector (Tamura *et al.* 2000). To date, we have successfully introduced several genes into silkworms, and we have used these transformants to analyze gene function and to elucidate physiological phenomena (S. Inoue and M. Imamura, unpublished data). We now wish to extend our studies in the silkworm to the adaptation of the GAL4/upstream activating sequence (UAS) system (Fischer *et al.* 1988; Brand and Perrimon 1993), which is a powerful technique for unraveling gene function. The *GAL4/UAS* system has been used routinely in Drosophila (Brand and Perrimon 1993) and has also been adapted to the mouse (Ornitz *et al.* 1991), zebrafish (Scheer and Campos-Ortega 1999), Xenopus (Hartley *et al.* 2002), and Arabidopsis (Guyer *et al.* 1998). This technique relies on the generation of transgenic lines that carry an activator or effector construct. The activator lines express the GAL4 yeast transcription factor under the control of a test promoter, whereas the effector lines contain the GAL4-binding sequence linked to the gene of interest (Brand and Perrimon 1993).

The *GAL4/UAS* system has certain advantages. First, it enables one to analyze simultaneously the effects of a single transgene selectively in different tissues and at different developmental stages. Conversely, it can also be used to study several different genes in a particular tissue or cell or at a specific time point. Second, this system makes possible the generation of transgenic lines that carry lethal genes or genes for toxic proteins and enables the functional analysis of these genes as well as the targeted destruction of a cell or tissue. Third, the *GAL4* system can be used to amplify the expression level of a transgene.

In this study, we demonstrate the feasibility of using the *GAL4/UAS* system in combination with the *piggyBac* transposon vector in the silkworm, by showing that the green fluorescent protein (GFP) gene is expressed in a predictable tissue-specific pattern in the progeny of crosses between the *GAL4* and *UAS-GFP* lines. This study emphasizes that the *GAL4* system using the *piggyBac* vector is also applicable to non-drosophilid insects that have undergone successful germ-line transformation with the *piggyBac* vector.

MATERIALS AND METHODS

Silkworm strains: The *w1-pnd* strain, which is nondiapausing and has nonpigmented eggs and eyes, was used in these experiments. The eggs of this strain develop to the larval stage, without termination of development, 11 days after the injection of DNA. The larvae were reared on an artificial diet (Nihon Nosanko) at 25°. This strain is maintained at the National Institute of Agrobiological Sciences.

Construction of vectors: The plasmids (Figure 1) were constructed as described below.

pBacUAS-GFP: pBacUAS-GFP was constructed from pPIG-A3DsRed1b, which was designed to identify organs and cells in the transplantation experiment. The *Bam*HI-*Not*I fragment of pPIGA3GFP (Tamura *et al.* 2000), which contains the EGFP sequence, was replaced with the *Bam*HI-*Not*I fragment from pDsRed1-N1 (CLONTECH, Palo Alto, CA), which contains the DsRed1 sequence, to yield the plasmid pPIGA3DsRed1a. To delete the polylinker sequence 5′-GAATTCGAGCTCGG TACCCGGGGATCCTCTAGA-3′, which contains *Eco*RI, *Sac*I, *Kpn*I, *Sma*I, *Bam*HI, and *Xba*I restriction sites, from vector pPIGA3DsRed1a, PCR was conducted with *pfu* DNA polymerase (Stratagene, La Jolla, CA) using pPIGA3DsRed1a plasmid DNA as the template. The nucleotide sequences of the primers were 5′-GGCGTCGACGTAATCATGGTCATAGCTGTTTCC-3′ (forward primer) and 5′-GCACGCGTTCGTGTACAGAC GTA-3′ (reverse primer). The PCR conditions were initial denaturation at 94° for 2 min, 30 cycles of 94° for 30 sec, 55° for 30 sec, and 72° for 3 min, followed by 72° for 5 min. The amplified fragment was digested with *Sal*I and *Mlu*I and then ligated with the 4.9-kb *Sal*I-*Mlu*I fragment derived from pPIGA3DsRed1a, which contained the *BmA3* promoter fragment and the *DsRed1* gene. The resulting plasmid was named pPIGA3DsRed1b. The *Hin*dIII-*Eco*RI fragment containing the UAS (the GAL4-upstream activating sequence) and TATA element of the *D. melanogaster* heat-shock protein 70 (Dmhsp70) promoter (Brand and Perrimon 1993) was subcloned into pEGFP-N1 (CLONTECH). The *UAS-EGFP* fragment was excised using *Xho*I and *Not*I and inserted into the *Not*I-*Xho*I site of pPIGA3DsRed1b to yield plasmid pBacUAS-GFP.

pBacBmA3-GAL4: The 647-bp fragment that lies upstream of the ATG start codon of the *B. mori* cytoplasmic actin A3 gene (BmA3) was amplified by PCR and used as the *BmA3* promoter. The nucleotide sequences of the primers were as follows: 5′-GGCGCGCCTCGAGCTCAAGCTTGATG-3′ (forward primer) and 5′-GGATCCCTTGAATTAGTCTGCAAG-3′ (reverse primer). The recognition sequences for *Asc*I and *Bam*HI were added to the forward and the reverse primer, respectively. PCR was conducted with LA Taq (Takara) using the pPIGA3GFP plasmid DNA as a template. The PCR cycling conditions were as follows: initial denaturation at 95° for 2 min, 30 cycles of 95° for 30 sec, 55° for 30 sec, and 72° for 40 sec, followed by 72° for 7 min. The amplified fragment was subcloned in the pGEM-T Easy vector (Promega, Madison,WI), and the constructs were digested with *Bam*HI and *Sac*II. The *Bam*HI-*Sac*II fragment, which contained the *GAL4* gene and *Dmhsp70* terminator that originated from pGaTB (Brand and Perrimon 1993), was inserted into the *Bam*HI-*Sac*II site of the pGEM-T Easy vector containing the *BmA3* promoter fragment. The fragment that contained the *BmA3-GAL4* gene was excised from this plasmid by digestion with *Not*I and blunt-end ligated into the *Hpa*I site of p3E1.2 that was the plasmid containing an intact *piggyBac* transposon element (Cary *et al.* 1989; Fraser *et al.* 1995).

pBac3xP3-GAL4: The 251-bp fragment that included the *3xP3* promoter was obtained by PCR using pBac[3xP3-EGFPafm] (Horn and Wimmer 2000) as the template. The nucleotide sequences of the primers were as follows: 5′-AATAT GCGAATTCGAGCTCGCCCGGGGATCTAATTC-3′ (forward primer) and 5′-TGCAGGAATTCGGGCCCGCGGTACCGTC GACTCTAGC-3′ (reverse primer). Single *Eco*RI sites were added to both primers. PCR was carried out as follows: initial denaturation at 95° for 2 min, 30 cycles of 95° for 30 sec, 55° for 30 sec, and 72° for 30 sec, followed by 72° for 7 min. The *3xP3* promoter fragment was subcloned into the pGEM-T Easy vector. The *Bam*HI and *Sac*II fragment that contained *GAL4* and the *Dmhsp70* terminator, which was excised from pGaTB,

was inserted into the *Bam*HI and *Sac*II sites of pBluescript II SK− (Stratagene). The *3xP3* promoter fragment was excised with *Eco*RI from the TA vector and inserted into the *Eco*RI site of the pBluescript II SK− derivative that already contained the *GAL4* gene and *Dmhsp70* terminator. The *3xP3-GAL4* fusion was removed as a *Not*I-*Eco*RV fragment from this plasmid and blunt-end ligated into the *Hpa*I site of p3E1.2.

pBacFiL-GAL4/3xP3-CFP: To amplify the 740-bp region upstream of the fibroin light chain (FiL) gene, PCR was conducted using the plasmid that contained the *FiL* gene (Kikuchi *et al.* 1992) as the template, using the following primers: 5′-GGCGCGCCTGCATATTGGACATCC-3′ (forward primer) and 5′-CGCGGATCCTTTAGTGGTCTGTTA-3′ (reverse primer). The *Asc*I and *Bam*HI sites were attached to the forward and the reverse primers, respectively. The PCR cycling conditions were as follows: initial denaturation at 95° for 2 min, 30 cycles of 95° for 30 sec, 55° for 30 sec, and 72° for 40 sec, followed by 72° for 7 min. The amplified fragment was subcloned into pGEM-T Easy, and the *Bam*HI-*Sac*II fragment of the *GAL4* gene from pGaTB was inserted into the *Bam*HI and *Sac*II site of this plasmid. The fragment that contained the *FiL-GAL4* gene was excised by digestion with *Not*I and blunt-end ligated into the *Hpa*I site of p3E1.2 from which superfluous *Eco*RI, *Sac*I, *Kpn*I, *Sma*I, and *Bam*HI sites were removed. The resultant plasmid was named pBacFiL-GAL4. Then, to introduce a transformation marker into pBacFiL-GAL4, the *3xP3-ECFP-SV40* terminator fragment was amplified by PCR using pBac[3xP3-ECFPafm] plasmid DNA as the template and the following primers: 5′-CAAGATCTAATTC GAGCTCGCCCGGGGATCTAATTC-3′ (forward primer) and 5′-TAGCAGATCTGTACGCGTATCGATAAGCTTTAAG-3′ (reverse primer). Both primers had *Bgl*II sites at their 5′-ends. PCR was performed as follows: initial denaturation at 95° for 2 min, 30 cycles of 95° for 30 sec, 55° for 30 sec, and 72° for 30 sec, followed by 72° for 7 min. The PCR product was digested with *Bgl*II and cloned into the *Bgl*II site of pBacFiL-GAL4.

All the PCR products and constructed plasmids were verified by sequencing using an ABI310 or ABI377 DNA sequencer and the BigDye termination DNA sequencing kit (PE Applied Biosystems, Foster City, CA).

Injection of DNA into embryos and detection of GFP and CFP fluorescence: Plasmid DNA for injection was purified using a plasmid purification kit (QIAGEN, Valencia, CA). pHA3PIG (Tamura *et al.* 2000) was used as the helper plasmid for the production of transposase. Vector and helper plasmids (each 0.2 μg/μl) were resuspended in 0.5 mm phosphate buffer (pH 7.0), 5 mm KCl, and injected into eggs that were collected between 3 and 5 hr after egg oviposition. In the transient assay, only the vector plasmids were injected. GFP and CFP (the spectral variant of GFP, cyan fluorescent protein) fluorescence was observed under a fluorescence microscope that was equipped with filter sets for GFP2 and CFP (Leica), respectively. Transient expression of the injected DNA was observed in the G_0 eggs 3 days after injection. Screening was performed at a late stage of embryonic development for transformants that were driven by the *3xP3* promoter and in the first instar larvae for transformants that carried the *BmA3* promoter.

Preparation of genomic DNA and Southern blot analysis: Genomic DNA was extracted from adult moths by the SDS-phenol method (Ohshima and Suzuki 1977). The DNA (4 μg) was digested with restriction enzymes and fractionated on an 0.8% agarose gel. *Xho*I and *Kpn*I were used to digest the genomic DNA of the *BmA3-GAL4*, *3xP3-GAL4*, and *UAS-GFP* strains, and *Bgl*II was used to digest the genomic DNA of the *FiL-GAL4* strain. The DNA samples were transferred to a Hybond-N+ nylon membrane (Amersham Pharmacia Biotech) and fixed by UV cross-linking. Hybridization was performed using the Alkphos direct labeling and detection system (Amersham Pharmacia Biotech). The probes for the *GAL4* and *GFP* genes were prepared from the ~2500-bp *Cla*I fragment of pGaTB and the ~1200-bp *Xho*I-*Not*I fragment of pBac UAS-GFP, respectively.

PCR detection of transgenes: To distinguish larvae with single *GAL4* or *GFP* genes from the GFP-negative G_2 larvae, PCR was carried out using 50 ng of genomic DNA from the hemocytes of a single larva as the template. Genomic DNA was prepared using the DNeasy tissue kit (QIAGEN). The following primers were used for gene detection: for the *GFP* gene, 5′-CTCGTCCTTCAGTGATAGCAG-3′ (forward) and 5′-CGCTTAACATGATGGAGCATCG-3′ (reverse) and for the *GAL4* gene, 5′-CACATGAAGCAGCACGACTTCTTC-3′ (forward) and 5′-CTTGATGCCGTTCTTCTGCTTGTC-3′ (reverse). PCR was carried out as follows: initial denaturation at 95° for 2 min, 30 cycles of 95° for 30 sec, 63° for 30 sec, and 72° for 30 sec, followed by 72° for 7 min.

RESULTS

***Trans*-activation of the *UAS-GFP* gene by the *GAL4* promoter element in silkworm embryos in a transient expression assay:** To investigate whether the *GAL4/UAS* system worked in the silkworm, we first performed a transient expression assay in the embryos. To date, three promoters have been reported to work in transgenic silkworms: the *B. mori* cytoplasmic actin promoter (*BmA3*; Tamura *et al.* 2000), the artificial *3xP3* promoter (Thomas *et al.* 2002), and the promoter of the *D. melanogaster heat-shock protein 70* gene (*Dmhsp70*; Uhlirova *et al.* 2002). The *BmA3* promoter has been used to drive gene expression in many types of cells at all developmental stages, and the *3xP3* promoter has been shown to stimulate the expression of introduced genes in the cells of stemmata and compound eyes, as well as certain cells of the CNS (Horn *et al.* 2000). We constructed two GAL4 driver plasmids, which were under the control of the *BmA3* and *3xP3* promoters (pBacBmA3-GAL4 and pBac3xP3-GAL4), and a UAS reporter plasmid that contained the *UAS-GFP* fusion gene (pBacUAS-GFP; Figure 1). High levels of GFP expression were observed following the injection of either pBacBmA3-GAL4 or pBac3xP3-GAL4 with pBacUAS-GFP into the embryos (Figure 2). GFP fluorescence was not generated when the plasmids were injected independently. These results showed that transactivation of the *UAS-GFP* gene by GAL4 occurred during transient expression in silkworm embryos.

The GFP expression of eggs that were co-injected with pBacBmA3-GAL4 and pBacUAS-GFP was much stronger than that of eggs that were injected with pPIGA3GFP that have the *GFP* gene under direct control of the *BmA3* promoter. Similarly, eggs that were co-injected with pBac3xP3-GAL4 and pBacUAS-GFP also showed high levels of GFP expression, although the GFP fluorescence was poor when a single *3xP3* promoter construct, pBac[3xP3-EGFPafm], was injected into the silkworm embryos (data not shown). These results suggest

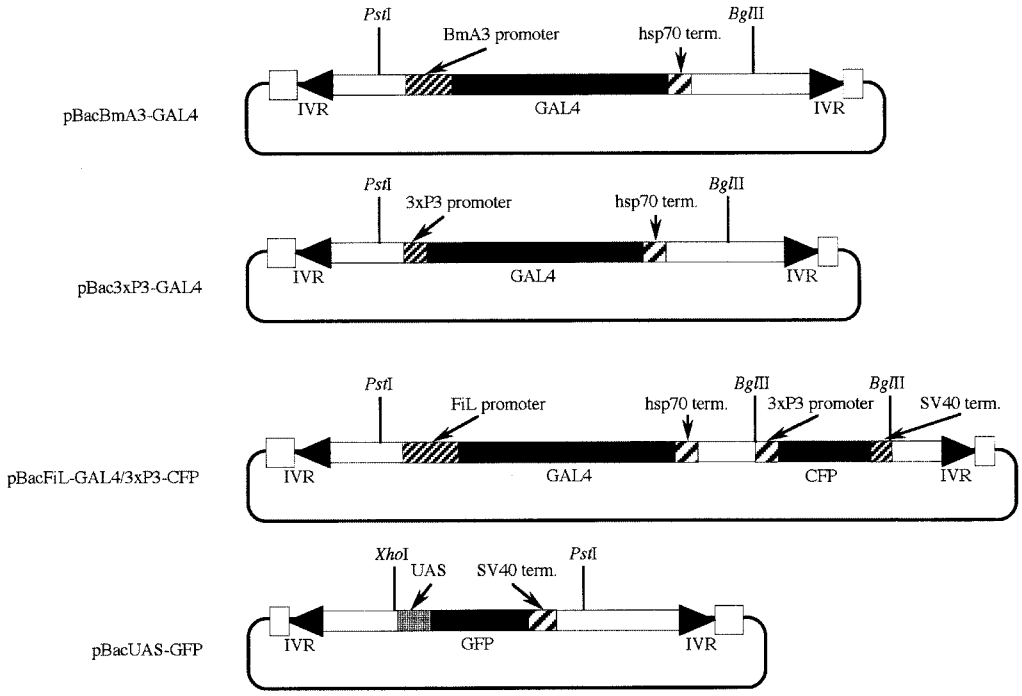

FIGURE 1.—Organization of the GAL4 and UAS constructs derived from the *piggyBac* transposon element. The GAL4 promoter fragments were inserted into the *Hpa*I site of p3E1.2. The *3xP3-CFP* fragment was inserted into the *Bgl*II site to produce pBacFiL-GAL4/3xP3-CFP. pBacUAS-GFP was constructed from pPIG-A3GFP.

that the regulation of expression by the *BmA3* and *3xP3* promoters is enhanced in the *GAL4/UAS* system.

BmA3-GAL4 and 3xP3-GAL4 both drive the expression of the *UAS-GFP* gene in transgenic silkworms: Next, we carried out experiments to show that the *GAL4/UAS* system functioned in transgenic silkworms (Figure 3). When we started this study, only two promoters (*BmA3* and *3xP3*) and one fluorescent marker (GFP) had been reported to function in transgenic silkworms. Therefore, we developed the following strategy to show that the *GAL4/UAS* system applies to the silkworm. First, we established transformants that carried both the *promoter-GAL4* and *UAS-GFP* genes with no marker gene for transformation. If these transformants produce GFP fluorescence, then the *GAL4/UAS* system functions in the silkworm. However, it is also necessary to prove that transactivation by GAL4 occurs when *GAL4* and the *UAS-GFP* gene coexist as a result of mating. Therefore,

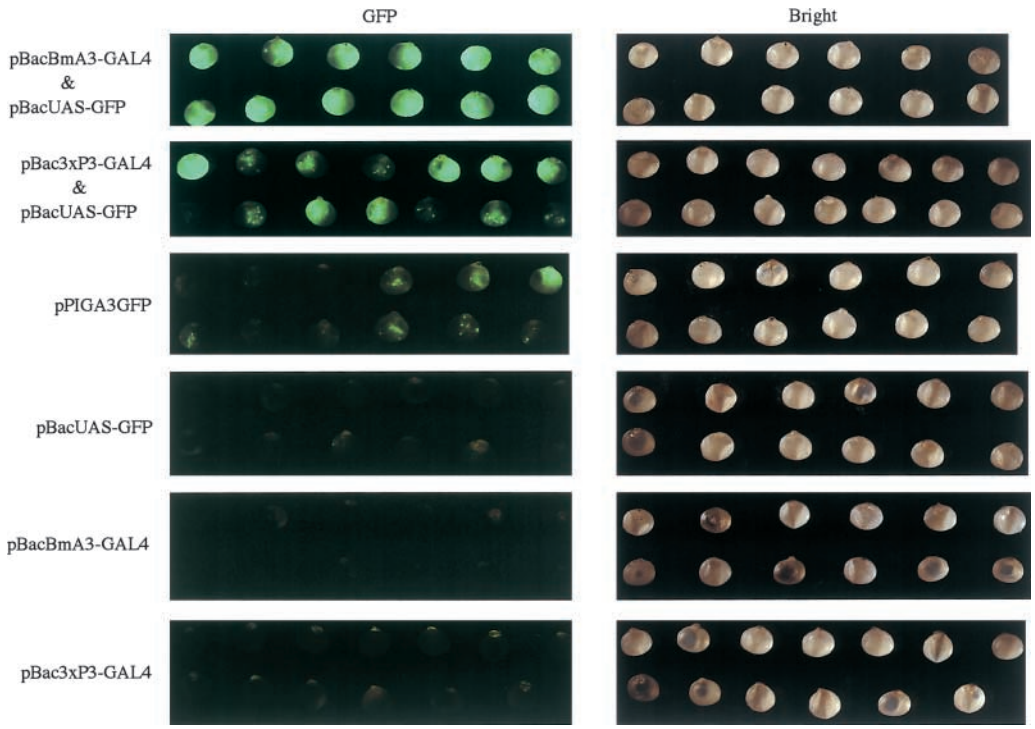

FIGURE 2.—Transient expression of the *GFP* gene in embryos using the GAL4 and UAS constructs. (Left) GFP-fluorescent image of eggs that were injected with DNA constructs; (right) corresponding bright-field image. The plasmids (each 200 μg/μl) were injected into 3- to 4-hr-old embryos, and the embryos were observed 3 days after injection.

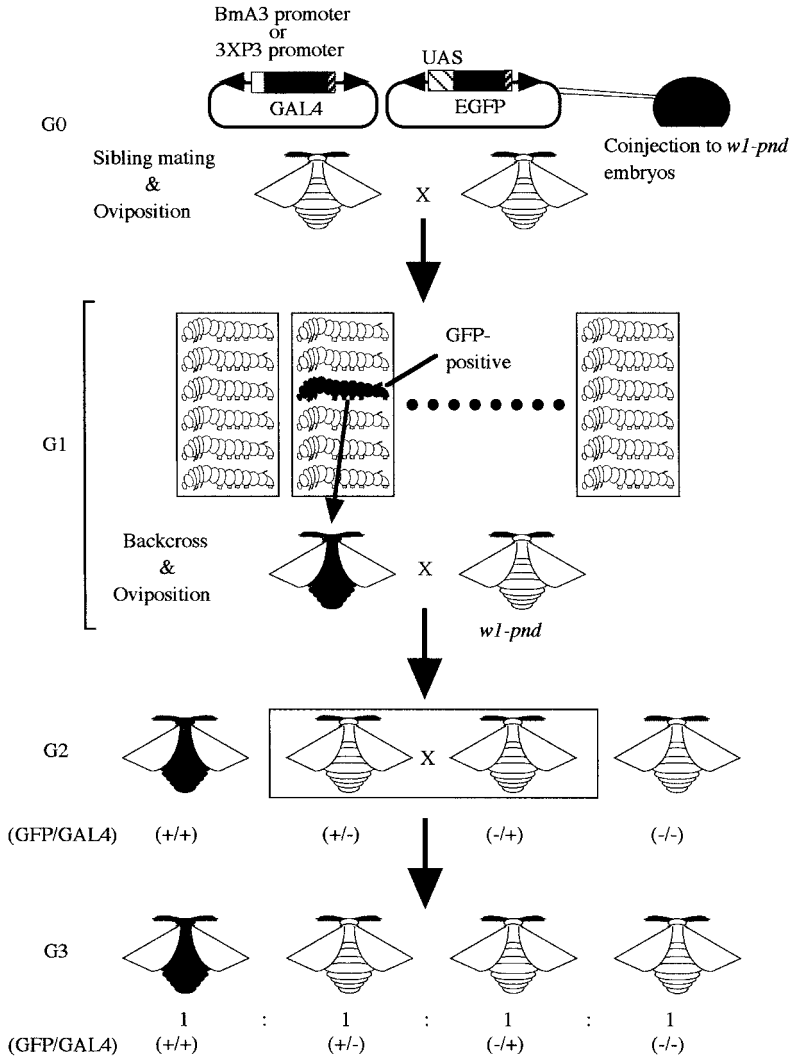

FIGURE 3.—System for testing the *GAL4/UAS* system in *B. mori*. First, the promoter-GAL4 and *UAS-GFP* plasmid vectors were co-injected into embryos so that no transformation marker was needed. If the *GAL4/UAS* system functions properly in the transgenic silkworms, then GFP-positive animals should be recovered in G_1. Next, a crossing experiment was performed to confirm that the *GAL4/UAS* system can function when *GAL4* and *UAS* coexist as a result of crossing.

crossing experiments were done to recover transformants with only the *GAL4* or *UAS-GFP* gene. GFP-positive G_1 animals were backcrossed to the *w1-pnd* strain, to generate GFP-negative G_2 animals with only the *GAL4* or *UAS-GFP* gene. Then, the *GAL4* and *UAS* lines were crossed, because if GFP-positive G_3 animals emerged in the ratio of one to three this would prove that the *GAL4/UAS* system applies to the silkworm transgenic system.

The pBacBmA3-GAL4 construct was injected, along with pBacUAS-GFP and the pHA3PIG helper plasmid as a source of transposase, into ~1500 eggs of the *w1-pnd* strain. About 270 G_0 fertile adults were recovered, and they were sibling mated to decrease the number of broods for screening. As a result of screening of 112 broods, 3 broods with GFP-positive larvae were identified (2.7%; Table 1). Similarly ~1800 eggs were injected with pBac3xP3-GAL4. After sibling mating of ~270 G_0 adults, 121 broods were obtained, and 3 broods with GFP-positive larvae were identified (2.5%). In any GFP-positive individuals, GFP fluorescence was observed in tissues in which both promoters were expected to drive *GAL4* gene expression (Figure 4). The frequency of G_1 GFP-positive larvae in the broods from G_0 moths that were injected with the two plasmids, pBacBmA3-GAL4 and pBacUAS-GFP, was between 0.4 and 2.1%; it was between 0.4 and 17.7% for broods from G_0 moths that were injected with the pBac3xP3-GAL4 and pBacUAS-GFP. Unfortunately, the G_1 GFP-positive animals in brood 3 produced by mating moths injected with pBacBmA3-GAL4 and pBacUAS-GFP and brood 1 produced by mating moths injected with pBac3xP3-GAL4 plus pBacUAS-GFP were lost before they became moths.

Southern blot analysis was performed on the genomic DNAs of transformed G_1 animals to identify differences in the insert positions and copy numbers of the transgenes. Five fertile GFP-positive adults in broods 1 and 2, whose parents were injected with pBacBmA3-GAL4 and pBacUAS-GFP, were found to carry single copies of the *GAL4* and *UAS-GFP* genes (Figure 5). The banding patterns were identical for all the transformants (data not shown), which indicated that all of the transformants that carried the *BmA3-GAL4* and *UAS-GFP* genes were produced from the same parent. The finding that two different broods possess the same insertion

TABLE 1

Injection and transformation of GAL4 and UAS vectors (A) and study of GFP-positive transgenic animals (B)

A.	No. of injected eggs	No. of hatched eggs (%)	No. of fertile adults (%)	Total no. of G_0 broods	No. of G_0 broods with GFP-positive animals (%)
pBacBmA3-GAL4 + pBacUAS-EGFP	1502	400 (26.6)	268 (17.8)	112	3 (2.7)
pBac3xP3-GAL4 + pBacUAS-EGFP	1760	413 (23.5)	271 (15.4)	121	3 (2.5)

B.	Brood	No. of G_1 hatched eggs	No. of GFP-positive animals (%)	No. of fertile adults
BmA3-GAL4	1	379	8 (2.1)	3
+ UAS-EGFP	2	321	2 (0.6)	2
	3	235	1 (0.4)	0
3xP3-GAL4	1	229	1 (0.4)	0
+ UAS-EGFP	2	333	59 (17.7)	27
	3	379	53 (14.0)	41

can be explained by the fact that the G_0 males were repeatedly mated with females because of the limited number. The line that contained the *BmA3-GAL4* and *UAS-GFP* genes is referred to as the *A3* line. Twenty-seven and 41 G_1 fertile adults with pBac3xP3-GAL4 and pBacUAS-GFP were recovered from GFP-positive broods 2 and 3, respectively. Southern blot analysis was carried out on the genomic DNA samples of 24 adults from each brood (data not shown). In brood 2, we found two types of transformant with single *GAL4* and *UAS-GFP* genes inserted at different positions, which we refer to as the *P2-1* and *P2-2* lines, respectively. On the other hand, there were three patterns of integration in brood 3. Although all of the transformants from brood 3 carried an identical single insertion of the *GAL4* gene, the *UAS-GFP* gene appeared in three different patterns: two patterns had single insertions at independent sites and the remaining pattern contained both inserts (Figure 5). We designate these lines as the *P3-1*, *P3-2*, and *P3-3* lines, respectively.

To recover animals that contained only the *promoter-GAL4* gene or only the *UAS-GFP* gene, we backcrossed the G_1 transformants with the *w1-pnd* host strain. The ratio of the GFP-positive and negative G_2 first instar larvae in all crosses was 1:3 (Table 2). Twenty-four GFP-negative fifth instar larvae were chosen randomly from each line, genomic DNA was prepared from the hemocytes of these animals, and PCR was performed using the *GAL4* and *GFP* gene-specific primers to check their genotypes (Figure 6). Thus, we obtained individuals with either a single *GAL4* or *UAS-GFP* gene. Although the segregation ratios of the genotype varied widely in

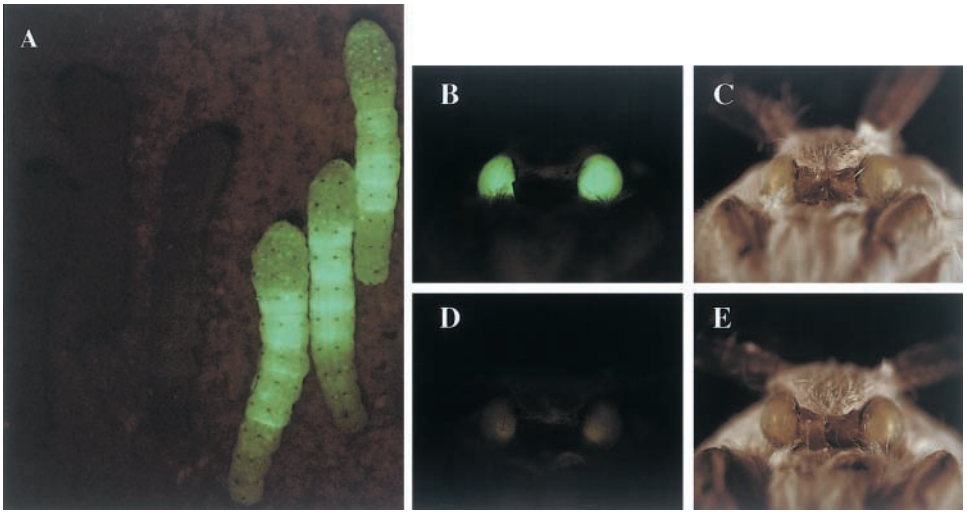

FIGURE 4.—Transgenic silkworms expressing the *GFP* gene under the control of the *GAL4/UAS* system. (A) Fluorescent images of 3-day-old first instar larvae that carry the *BmA3-GAL4* and *UAS-GFP* genes (right) and host-strain *w1-pnd* larvae as controls (left). (B) Fluorescent and (C) bright-field images of an adult that carries the *3xP3-GAL4* and *UAS-GFP* genes. (D) Fluorescent and (E) bright-field images of an adult *w1-pnd* moth as the control.

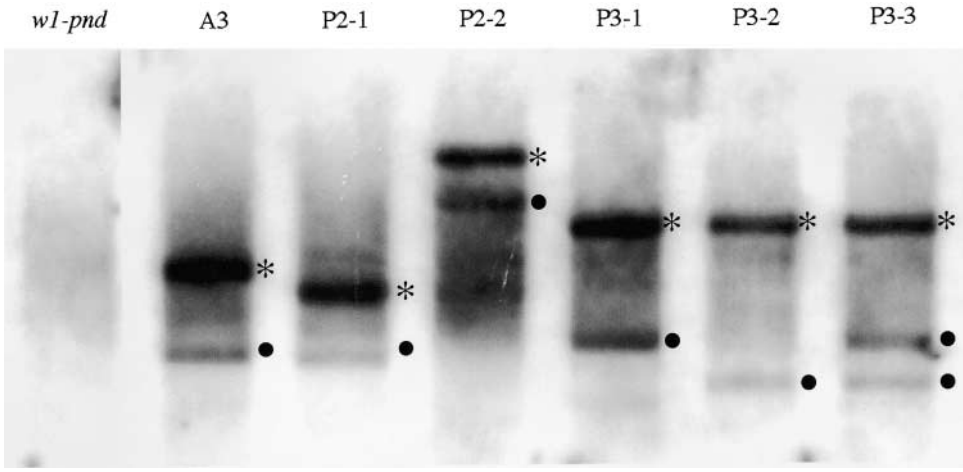

FIGURE 5.—Southern blot analysis of transgene integration patterns in G_1 GFP-positive silkworms. Genomic DNA samples from G_1 GFP-positive and *w1-pnd* adults were digested with *Xho*I and *Kpn*I, separated by agarose gel electrophoresis, and hybridized with *GAL4*- and *GFP*-specific probes. The individual DNA hybridization patterns of the *w1-pnd*, *A3*, *P2-1*, *P2-2*, *P3-1*, *P3-2*, and *P3-3* lines are shown. Asterisks and solid circles denote the signals for the *GAL4* and *GFP* probes, respectively.

24 investigated GFP-negative larvae, they were shown to fit a 1:1:1 ratio by chi-square statistical analysis (Table 2). This result suggested that the *GAL4* and *UAS-GFP* genes were dispersed throughout the transgenic chromosomes.

Moths from the four *GAL4* lines (one with the *BmA3-GAL4* gene from the *A3* line and three with the *3xP3-GAL4* gene from the *P2-1*, *P2-2*, and *P3-1* lines, respectively) were crossed with the *UAS-GFP* line that carried a single *UAS-GFP* gene from the *A3* line. In the offspring (G_3), ~25% of the larvae had acquired GFP-dependent fluorescence, whereas both parents were GFP negative, and the segregation ratio of the genotypes was 1:1:1:1 (Table 3). Southern blot analysis of genomic DNA samples of the G_3 GFP-positive individuals showed that all of them carried both the *GAL4* gene and the *UAS-GFP* gene from the G_2 lines (Figure 7). These results demonstrate that the *GAL4/UAS* system functions in the silkworm, even when the *GAL4* and *UAS-GFP* genes coexist after crossing.

Evaluation of the *GAL4/UAS* system in the transgenic silkworm using the fibroin L-chain promoter: We generated a *GAL4* line that carried *GAL4* gene driven by a promoter derived from the *FiL* gene and the *3xP3-CFP* gene as a fluorescent transformation marker. We then investigated the utility of the *3xP3-CFP* marker and whether the *FiL* promoter specifically drives gene expression via the *GAL4/UAS* system in the posterior division of the silk gland (PSG). The pBacFiL-GAL4/3xP3-CFP construct (Figure 1) was injected with helper plasmid DNA into 1006 eggs, and 19 broods with CFP-positive individuals were obtained (Table 4A; Figure 9, A and B). Adult moths from three different CFP-positive broods were backcrossed with the *w1-pnd* strain and established as the *FiL1*, *FiL2*, and *FiL3* lines. Southern analysis of the G_2 progeny showed that the *FiL1* and *FiL2* lines each had a single copy of the *GAL4* gene and that the *FiL3* line contained two copies of the gene (Figure 8). We found two copies of the *GAL4* gene in 12 individuals of the *FiL3* line (data not shown), suggesting they were tightly linked. Although the transgenic first instar larvae had five CFP-fluorescent stemmata (Figure 9, C and D), GFP fluorescence was not detected (Figure 9, E and F; middle). We then crossed these *GAL4* lines with the *UAS-GFP* line, which was heterozygous for the transgene (Figure 8). The ratio of the

TABLE 2

Segregation ratios in G_2 progeny after backcrossing GFP-positive G_1 with *w1-pnd*

G_1 genotype	Line	No. of G_2 hatched eggs	No. of GFP-positive animals (%)	Genotype of 24 investigated GFP-negative larvae (GAL4/GFP) (+/−)	(−/+)	(−/−)		*P* value (1:1:1)
A3GAL4 + UAS-EGFP	A3	247	59 (23.9)	4	7	13	24	0.07
3xP3GAL4 + UAS-EGFP	P2-1	441	107 (24.3)	9	12	3	24	0.07
	P2-2	414	106 (25.6)	6	13	5	24	0.09
	P3-1	345	74 (21.4)	6	7	11	24	0.42

The genotype was determined by PCR using genomic DNA from larval hemocytes. The expected segregation ratio was 1:1:1, and *P* values based on the chi-square test were $P > 0.05$ in all crosses.

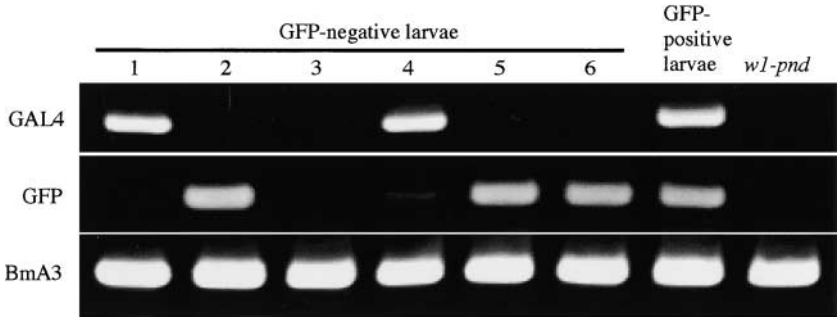

FIGURE 6.—PCR screening of G_2 transformants that carried single *GAL4* or *UAS-GFP* genes. Genomic PCR was conducted to identify individuals that carried a single *GAL4* or *UAS-GFP* gene. Part of the screening process is shown. Genomic DNA was prepared from hemocytes of GFP-negative fifth instar larvae that were derived from a backcross of GFP-positive G_1 with *w1-pnd*. PCR was carried out using the *GAL4-*, *GFP-*, and actin A3-specific primers with genomic DNA as the template. Each lane shows the PCR product from a single larva.

CFP-positive, CFP/GFP-positive, and negative larvae in all crosses was 1:1:2 (Table 4B). This result supports the notion that the *GAL4* genes in the *FiL3* line were tightly linked. In the progeny of these crosses, ~25% of the larvae gave strong GFP fluorescence on the side where the silk glands were located (Table 4B; Figure 9, E and F). Subsequently, the silk glands were dissected from 5-day-old fifth instar larvae and observed with a fluorescent microscope. Very strong GFP fluorescence was detected in the PSG of all the GFP-positive individuals (Figure 9, G–J), but not in GFP-negative individuals (Figure 9, K and L). Interestingly, the PSG in the *FiL1*, *-2*, and *-3* lines was shortened and appeared knotted (Figure 9, G and H), while the PSG in the other line was normal (Figure 9, I and J). This abnormality in PSG was thought to be caused by cell deformation.

DISCUSSION

In this study, we successfully constructed the *GAL4/UAS* system in the transgenic silkworm and showed that it could be used to express the *GFP* gene. The transgenes were normally inherited in a Mendelian manner in all of the *GAL4* lines (*BmA3-GAL4*, *3xP3-GAL4*, *FiL-GAL4*) and in the *UAS-GFP* line, which indicated that the viability of these lines was not affected by the expression of the transgene. These findings demonstrate that the *GAL4/UAS* system can be used for targeted transgene expression in silkworms. To date, the *GAL4/UAS* system has been shown to function in *D. melanogaster* (FISCHER *et al.* 1988; BRAND and PERRIMON 1993), mice (ORNITZ *et al.* 1991), Arabidopsis (GUYER *et al.* 1998), zebrafish (SCHEER and CAMPOS-ORTEGA 1999; KOSTER and FRASER 2001), and frogs (HARTLEY *et al.* 2002). Our study represents the first attempt to show that the *GAL4/UAS* system is applicable to non-drosophilid insects. Recently, *piggyBac*-mediated germ-line transformation has been used successfully in various insects, such as the medfly, *Ceratitis capitata* (HANDLER *et al.* 1998); the red flour beetle, *Tribolium castaneum* (BERGHAMMER *et al.* 1999); the pink bollworm, *Pectinophora gossypiella* (PELOQUIN *et al.* 2000); the Oriental fruit fly, *Bactrocera dorsalis* (HANDLER and MCCOMBS 2000); the Caribbean fruit fly, *Anastrepha suspensa* (HANDLER and HARRELL 2001); the housefly, *Musca domestica* (HEDIGER *et al.* 2001); the yellow fever mosquito, *Aedes aegypti* (KOKOZA *et al.* 2001); the malaria mosquito, *Anopheles stephensi* (NOLAN *et al.* 2002); and the Australian sheep blowfly, *Lucilia cuprina* (HEINRICH *et al.* 2002). Therefore, the *GAL4/UAS* system with the *piggyBac* vector should also be applicable to these insects.

TABLE 3

Segregation ratios in the progeny after crossing *GAL4* lines with the *UAS-GFP* line

GFP fluorescence	Genotype	A3	P2-1	P2-2	P3-1
+	*GAL4/GFP*	11	10	13	14
−	*GAL4*	12	11	10	12
−	*GFP*	10	12	8	8
−	None	15	15	17	14
Total		48	48	48	48
	P value (1:1:1:1):	0.76	0.76	0.28	0.57

The expected segregation ratio was 1:1:1:1, and *P* values based on the chi-square test were $P > 0.05$ in all crosses.

In the transgenic silkworm *B. mori*, the actin A3 (*BmA3*) and artificial *3xP3* promoters had been used to drive the expression of the *GFP* gene (TAMURA *et al.* 2000; THOMAS *et al.* 2002). In this study, we initially used the *BmA3* and the *3xP3* promoter to drive the *GAL4* gene and showed that these promoters produced sufficient amounts of GFP for imaging purposes. However, the GFP expression level in the transgenic silkworm did not reflect precisely the levels of amplified expression in transient assays of the embryos (Figure 2). It has been reported that transgene expression by GAL4 is somewhat weaker than expected in transgenic zebrafish (SCHEER and CAMPOS-ORTEGA 1999) and transgenic Xenopus (HARTLEY *et al.* 2002). To increase transgene expression via the *GAL4/UAS* system in silkworms, the following modifications may be useful: (1) the introduction of an insulator to both the *GAL4* and *UAS* constructs (BAROLO *et al.* 2000); (2) the insertion of several copies of the UAS-linked gene of interest into the chromosomes (KOSTER and FRASER 2001); and (3) the use of the GAL4-VP16 protein, which is a fusion of the GAL4 DNA-binding domain and the herpes simplex virus tran-

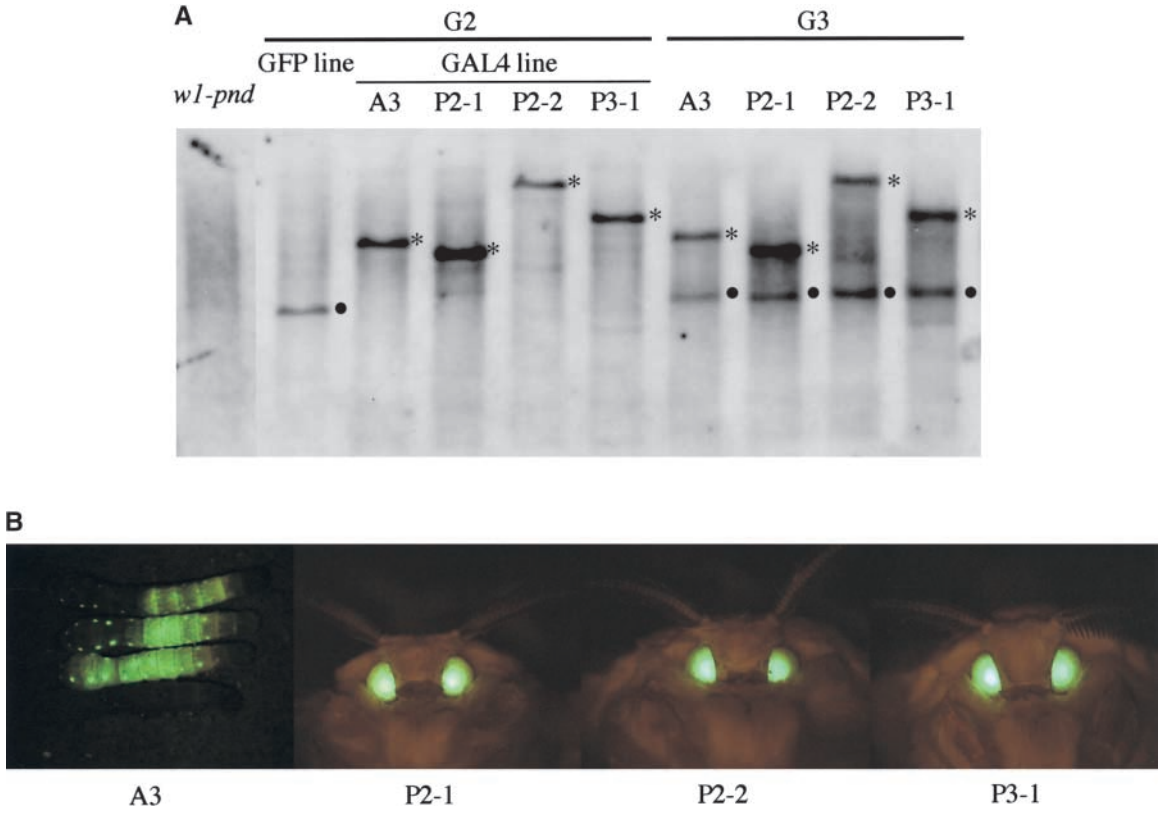

FIGURE 7.—Analysis of G_3 GFP-positive animals. (A) Southern blot analysis of G_3 GFP-positive animals and of G_2 individuals that carry a *GAL4* or *UAS-GFP* gene. A3, P2-1, P2-2, and P3-1 indicate the origin of the *GAL4* gene borne by each individual. Asterisks and solid circles denote the DNA fragments that hybridized with the *GAL4* and *GFP* probes, respectively. Genomic DNA was double digested with *Xho*I and *Kpn*I. (B) Fluorescent images of the G_3 GFP-positive animals. In A3, the three upper larvae are transformants that carry both the *BmA3-GAL4* and the *UAS-GFP* genes, and the lower larva is a *w1-pnd* individual. P2-1, P2-2, and P3-1 show moths that carry the *UAS-GFP* gene plus the *3xP3-GAL4* gene that originated in the *P2-1*, *P2-2*, or *P3-1* lines, respectively.

scriptional-activation domain VP16 (SADOWSKI *et al.* 1988).

The transformation efficiency of the *FiL-GAL4* line was ~11% (as a percentage of all the G_0 broods). This value is much higher than that reported previously for transgenic silkworms (TAMURA *et al.* 2000; THOMAS *et al.* 2002; UHLIROVA *et al.* 2002). This is probably due to an improved injection method (T. TAMURA, G. X. QUAN, T. KANDA and N. KUWABARA, unpublished data). Recently, it has been reported that the mobilization

TABLE 4

Transformation of GAL4 vector carrying the fibroin L-chain promoter (A) and segregation of phenotypes in the progeny after crosses between *FiL-GAL4* lines and the *UAS-GFP* line (B)

A. No. of injected eggs	No. of hatched eggs (%)	No. of fertile adults (%)	Total no. of G_0 broods	No. of G_0 broods with GFP positive (%)	
1006	544 (54.1)	352 (35.0)	169	19 (11.2)	
B. Line	Total no. of eggs	No. of CFP-positive larvae (%)	No. of CFP/GFP-positive larvae (%)	No. of negative larvae (%)	*P* value (1:1:2)
FiL1	118	25 (21.3)	31 (26.5)	62 (53.0)	0.63
FiL2	138	32 (23.2)	34 (24.6)	72 (52.2)	0.85
FiL3	87	20 (23.0)	19 (21.8)	48 (55.2)	0.62

The expected segregation ratio was 1:1:2, and *P* values based on the chi-square test were $P > 0.05$ in all crosses.

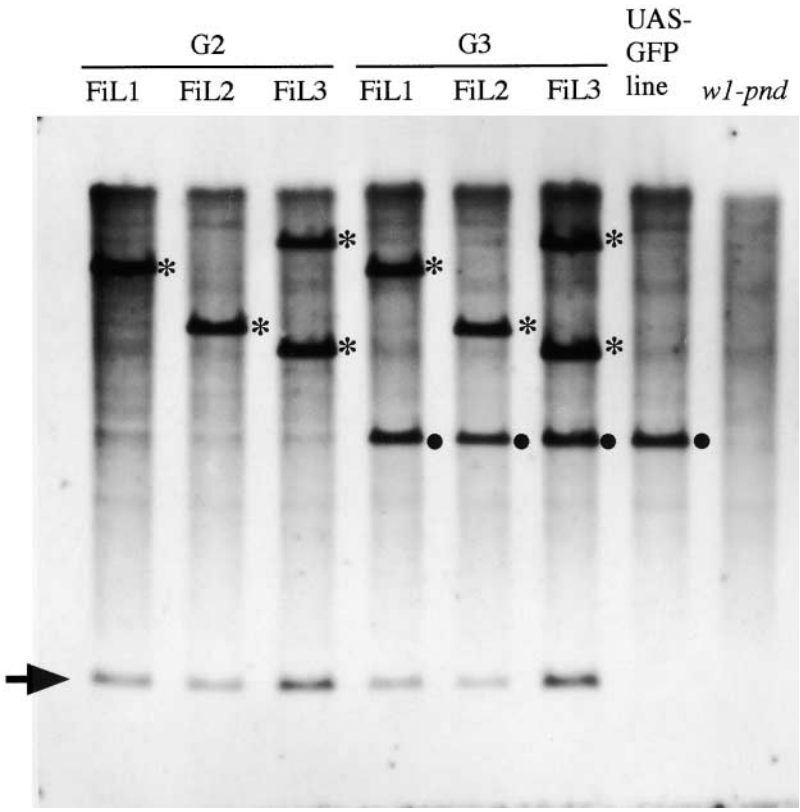

FIGURE 8.—Southern blot analysis of *FiL-GAL4* lines. Genomic DNA was digested with *Bgl*II. *FiL1*, *FiL2*, and *FiL3* in G_2 indicate the individuals that were used in crosses with the *UAS-GFP* line, and *FiL1*, *FiL2*, and *FiL3* in G_3 represent the GFP-positive individuals that resulted from these crosses, respectively. Asterisks and solid circles denote the DNA fragments that hybridized with the *GAL4* and *GFP* probes, respectively. The arrow shows the *3xP3-CFP* fragment that was excised from the GAL4 constructs by digestion with *Bgl*II. The signal for the *3xP3-CFP* fragment is stronger in the *FiL3* line than in the two other lines because this line has two copies of the *GAL4* gene.

frequency of the *Minos* transposable vector using *in vitro* synthesized mRNA as the source of transposase is 10-fold higher than that obtained using a helper plasmid (KAPETANAKI *et al.* 2002). This suggests that the application of *in vitro* synthesized *piggyBac* transposase mRNA may further increase the efficacy of *piggyBac*-mediated transformation of silkworms.

Although it has been reported that CFP fluorescence driven by the *3xP3* promoter in *D. melanogaster* is weaker than the fluorescence of GFP and YFP (the spectral variant of GFP, yellow fluorescent protein; HORN and WIMMER 2000), we used this as a marker in the generation of *FiL-GAL4*-transgenic silkworm lines. Indeed, the CFP-fluorescence intensity of the *FiL-GAL4* lines was weaker than that of GFP fluorescence in animals that were transformed with pBac[3xP3-GFPafm] (data not shown). However, screening with the *3xP3-CFP* gene is possible during late embryonic stages when the signal is weakest at all the stages, since both the eyes and the eggs of the *w1-pnd* strain are nonpigmented. Therefore, the *3xP3-CFP* gene can be used as a transformation marker, at least in this strain. However, when the stemmata and compound eyes of CFP-positive animals were observed with the GFP2 longpass filter set (Leica), it was difficult to distinguish CFP-positive animals from transgenic animals that carried the *3xP3-GFP* gene (data not shown). Thus, care is needed in identifying the tissues and organs that express the reporter gene when CFP and GFP are used as marker and reporter, respectively, in the same individual. CFP and DsRed2 or CFP and YFP are considered to be good marker combinations because these have well-separated excitation and emission spectra (HORN *et al.* 2002).

Abnormal PSGs that expressed GFP were observed in larvae of the three *FiL-GAL4* lines that were used in some experiments (*FiL1*, *-2*, and *-3* lines; Figure 9, G and H). This abnormality was also observed in larvae that carried only the *FiL-GAL4* genes (data not shown), which suggests that it was caused by GAL4 production. Furthermore, these lines formed no cocoon or a very thin-layer cocoon that resembled those formed in the fibroin-secretion-deficient mutants *Nd-s*^{D} and *Nd-s* (TAKEI *et al.* 1987; MORI *et al.* 1995; data not shown). On the other hand, one *FiL-GAL4* line formed normal PSG and a cocoon that was only slightly thinner than that of the wild type. The difference between normal and abnormal *FiL-GAL4* lines may be due to differences in the expression levels of the *GAL4* gene, as evidenced from the comparison of the relative GFP intensities (Figure 9, G–J). Approximately 200–300 cells in the PSG are specialized for mass production and secretion of the fibroin H- and L-chain proteins together with fibrohexamerin (P25; AKAI 1976; TANAKA *et al.* 1993; INOUE *et al.* 2000). Although it is known that GAL4 can be expressed in many cells and tissues of transgenic animals without any toxic effects, it is possible that the mass production of GAL4 in a tissue that is so highly specialized for protein production can be especially disruptive.

Various gene analysis systems using the *GAL4/UAS* system have been developed in Drosophila. These in-

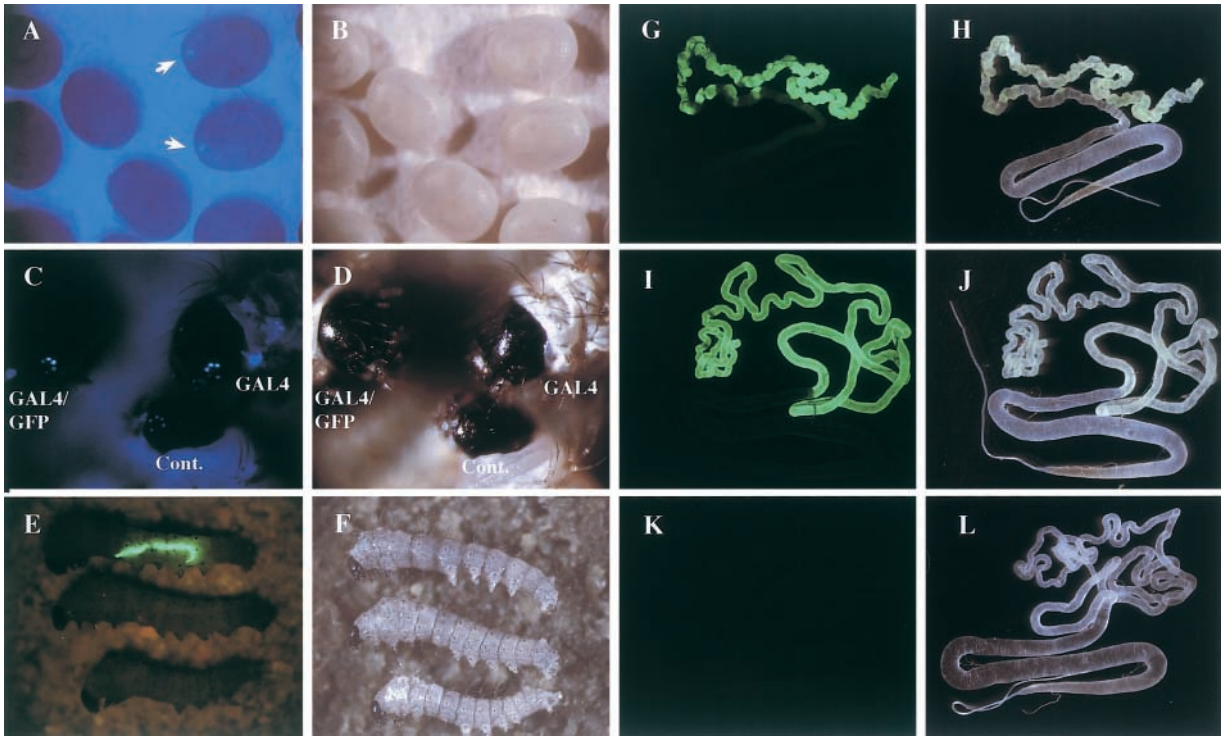

FIGURE 9.—Fluorescent images of transgenic silkworms that carry the *FiL-GAL4* gene. (A and B) Seven-day-old embryos of transformants that carry the *FiL-GAL4* gene with *3xP3-CFP* as a marker and nontransformants (A, CFP-fluorescent image; B, bright-field image). Arrows indicate the developing larval stemmata of the transformants. (C and D) The heads of 2-day-old first instar larvae that carry both the *FiL-GAL4/3xP3-CFP* and *UAS-GFP* genes (GAL4/GFP); only the *FiL-GAL4/3xP3-CFP* gene (GFP) and *w1-pnd* (Cont.) are shown (C, CFP-fluorescent image; D, bright-field image). (E and F) Two-day-old first instar larvae (E, GFP-fluorescent image; F, bright-field image). Top, transformant that carries both the *FiL-GAL4/3xP3-CFP* and *UAS-GFP* genes; middle, transformant that carries only the *FiL-GAL4/3xP3-CFP* gene; bottom, nontransformant. (G–L) Silk glands of transgenic fifth instar larvae that carry the *FiL-GAL4/3xP3-CFP* and *UAS-GFP* genes (G–J) and a *w1-pnd* larva (K and L; G, I, and K, GFP-fluorescent images; H, J, and L, bright-field images). G and H are the silk gland from an abnormal SG line (*FiL1* line), and I and J are the silk gland from a normal SG line. High-level GFP expression is observed only in the PSG of the transformants, which appear green even under bright-field microscopy.

clude enhancer trapping (BRAND and PERRIMON 1993), gain-of-function mutagenesis (RORTH 1996; TOBA *et al.* 1999), and gene silencing by interference using hairpin-loop RNA (KENNERDELL and CARTHEW 2000). In this study, we showed that the *GAL4/UAS* system is applicable to the silkworm. Recently it was shown that insertional mutagenesis and enhancer trapping is possible using the *piggyBac* vector in *D. melanogaster* (HORN *et al.* 2003). Therefore, in the near future we will be able to develop novel systems that employ the *GAL4/UAS* system for gene discovery and gene functional analysis in the silkworm. Once such systems are constructed, they will contribute to the advance of functional genomics for the silkworm and to comparative and functional genomics for lepidopteran species.

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